Optimisation of fungal cellulase production from textile waste using experimental design

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Abstract

Abundant textile waste raises increasing concerns worldwide in developing novel circular textiles approach. This study investigated the optimum cellulase production from textile waste by *Aspergillus niger* CKB. Textile wastes consisting of cotton and polyester in various ratios were used as low-cost feedstocks. Three types of cultivation media were compared in solid state fermentation, in which Mandels medium with yeast extract was selected due to their superior cellulase production. Conditions including moisture, pH, inoculum size and organic nitrogen were evaluated and optimised via Response Surface Methodology. Supplementary carbon sources and cellulase inducers were also employed to enhance the fungal growth and cellulase production. The results indicated that the optimised fermentation method significantly improved cellulase production efficiency and enzyme activity by 88.7% and 25.8%, respectively. The maximum cellulase activity reached 1.56 FPU g^{-1} in 6 days. The outcomes of this study led to efficient recovery of glucose and polyester, which could contribute to a closed-loop recycling strategy for the textile industry and enable the transition towards an advanced circular textiles economy.

Keywords: Cotton; Fungal cellulase; Response surface methodology; Solid state fermentation; Textile waste

1. Introduction

Globally increasing consumption of textiles and fashion products led to a huge accumulation of textile waste and serious environmental problems (Caulfield, 2009). In 2015-2016, the annually worldwide production volume of textile fibres reached 80-90 million tonnes and it is forecasted to exceed 100 million tonnes soon (Lenzing Corporation, 2016). The garment, textile and fashion industries are greatly pollutive and it is evaluated as the second most polluting industry in the world, following the petroleum industry (Sweeny, 2015). On the global average, 32 kg of textile wastes are discarded per capita each year, of which the majority (around 85%) is directly disposed by landfill or incineration, leading to soil contaminations and greenhouse gases emission (Textile Exchange, 2012). Waste & Resource Action Programme (WRAP, UK) evaluated that around 95% of landfilled textile waste is recyclable, whereas only 14-15% recycling rate has been reached (WRAP, 2016). Nowadays, textile recycling mainly relies on second-hand dumping and downcycling into rags, which actually do not capture values from textiles. In order to develop a new textiles economy based on a circular system, global textile manufacturers such as H&M are developing efficient recycling strategies to capture the embodied value of fibres (H&M, 2017).

In view of textile materials, cotton and polyester (PET) are the most widely used types of fibres. Approximately 35-40% of textile waste is comprised of cotton, which is a potential cellulosic feedstock for bioproducts, such as enzymes, ethanol and

biogas (Jeihanipour *et al*., 2010; Shen *et al.*, 2013). Pensupa *et al.* (2017) summarised the development of utilising textile waste through processes involving pretreatment, saccharification and fermentation. Lignocellulosic wastes (*e.g.* agricultural waste and horticultural waste) have been developed as low-cost substrates for cellulase production in the last decade (Bansal *et al.*, 2012; Xin and Geng, 2010). The feasibility of using cotton-based textile material in this area was investigated for the first time in our recent study (Hu *et al.*, 2017), which produced cellulase from textile waste (cotton and PET blends) by solid state fermentation (SSF).

Fermentation conditions of SSF are crucial for microbial growth and metabolic activity. There is a direct relationship between substrate, fermentation conditions and cellulase production (Yoon *et al*., 2014). As textile waste is a newly applied substrate in SSF, the suitable conditions deserve a comprehensive optimisation to maximise cellulase production. The affecting parameters in SSF include fermentation medium, temperature, moisture content, pH and supplementary nutrients (Sukumaran *et al*., 2005). Fermentation medium has been stressed in literature as it has profound effects on types and concentrations of fungal cellulase produced (Yoon *et al*., 2014). Moisture content of the medium is essential for fungal growth and metabolism in SSF, as well as affects the diffusion of nutrients and air. Low moisture content limits the solubility of nutrients while high moisture level could decrease the porosity of substrate and oxygen transfer (Kumar *et al*., 2011). Besides, as moisture requirement is directly related to the physical characters of substrate such as surface structure and water holding capacity, it is necessary to consider the nature of substrate applied in SSF when optimising the moisture condition (Orzua *et al*., 2009). Inoculum size is another crucial factor in enzyme production. At a low inoculum size, limited conidial cells cannot fully utilise the nutrients in medium, leading to poor cellulase

biosynthesis (Bansal *et al*., 2012). In contrast, excessive inoculum size usually causes nutritional imbalance and an anaerobic environment under tremendous fungal growth (Bansal *et al*., 2012). Other factors such as pH and temperature could also influence enzyme production and activity in SSF. The interactions between different variables should also be taken into account. For instance, the combination of moisture content and inoculum size plays a vital part in affecting the dynamics of microbial growth on solid substrates (*i.e.* colonisation of fungus, assembly of solid substrates), as well as mass and heat transfer in SSF (Ustok *et al*., 2007). In addition, supplementary carbon sources have been suggested to support fungal growth and to promote cellulase activity (Liang *et al*., 2012; Olsson *et al*., 2003). As an inducible enzyme, cellulase production can be initiated or enhanced by appropriate inducers under specific conditions, which allows a more controlled gene expression of the enzyme (Sukumaran *et al*., 2005).

Response Surface Methodology (RSM) is one of the most practical methods used in system optimisation (Kumar *et al*., 2011). Through experimental design and modelling, RSM not only identifies the effect of individual variables, but also evaluates the interaction of various parameters to seek the optimal solution. This method has been widely applied in optimisation studies of biotechnology and industrial processes (Kumar *et al*., 2011; Soleimaninanadegani *et al*., 2014). Levin *et al*. (2008) enhanced lignocellulosic enzyme production by optimisation of fermentation conditions through RSM, endo-xylanase activity was enhanced by 50.8%. Yasmeen *et al*. (2013) investigated lignocellulosic enzyme production using agricultural wastes with the cultivation conditions optimised by a five-factor-five-level Central Composition Design (CCD) model in RSM. The activities of lignin

peroxisase, manganese peroxidase and laccase were improved by 26.1%, 16.4% and 34.2%, respectively.

In order to develop a novel recycling method of textile waste, a recent research project funded by the Innovation Technology Commission in Hong Kong entitled "Textile Waste Recycling by Biological Method" is currently conducted by our group for recovery of cellulose and PET from blended materials. Based on our preliminary research of cellulase production from textile waste (Hu *et al.*, 2017), SSF conditions were systematically optimised via experimental design in this study. Different cultivation media and physical conditions were compared in terms of cellulase activity. Effect of supplementary carbon sources and presence of inducers on cellulase production were also investigated. Finally, an overall material balance of the bioconversion process was evaluated.

2. Materials and methods

2.1 Textile waste and microorganism

Three different types of cotton/PET blended textile wastes provided by H&M (Hennes & Mauritz, Far East) were used as feedstocks in this study. The cotton/PET blending ratios were 80/20, 60/40 and 40/60. The cellulase producing fungal strain *Aspergillus niger* CKB was obtained from Prof. Diannan Lu at Tsinghua University in China.

2.2 Chemicals and reagents

Sodium citrate buffer (50 mM, pH 4.8) and 3,5-dinitrosalicylic acid (DNS) solution were prepared according to the procedure illustrated by Adney and Baker (1996). Citric acid monohydrate, Rochelle salt (potassium sodium tartrate) and 3,5-dinitrosalicylic acid were purchased from Alfa Aesar (UK). Sodium hydroxide, potato starch and lactose monohydrate were supplied by VWR BDH Prolabo (UK). Sucrose (99.7%) and Avicel (cellulose microcrystalline, extra pure, a particle size of 90 μm) were ordered from Acros Organics (Belgium). Carboxymethylcellulose (sodium salt) and peptone were purchased from ChemCruz (USA) and UNI-CHEM (China), respectively.

2.3 Solid state fermentation (SSF)

Fungal cellulase was produced on textile waste via solid state fermentation. For each SSF, 2 g (dry weight) of textile waste scrap (0.8×0.8 cm^2) was mixed with 8 mL of cultivation medium. The pH of the fermentation medium was adjusted to the designated pH values (in the range of 4 - 8). After autoclaving at 121ºC for 15 min, the mixture was incubated with 0.3 mL of spore suspension (2×10^8 spores mL^{-1}) in a petri dish. SSF was conducted in an incubator for 9 days under static condition. The weight of each petri dish (with substrate and inoculum) was measured at the beginning of SSF and was maintained constant throughout fermentation by addition of deionization water (DI water) . Different temperature conditions (25-35°C) and initial moisture contents (55-95%) were employed. Each designed fermentation condition was tested in duplicate to obtain parallel results.

2.4 Selection of cultivation medium

Three different cultivation media were compared in this study: (i) Csiszar medium (Csiszar *et al*. 2007), (ii) Mandels medium with peptone (Mendels and Weber, 1969) and (iii) Mandels medium with yeast extract (YE). The compositions of these three media are listed in Table 1.

Three types of cotton/PET blended textile fabric were separately mixed with the three cultivation media. After autoclaving, the mixture was incubated with *A. niger* CKB (3×10^7 spores g^{-1}) under 75% moisture, pH 6.0 for 9 days at 28°C in duplicate. At the end of SSF, all samples were collected for cellulase activity assay.

Table 1. Compositions of different cultivation media used in SSF on textile waste.

Substance (unit: g L^{-1})	Csiszar medium	Mandels medium (with peptone)	Mandels medium (with YE)
Tween 80	-	1	1
Peptone	-	2.5 w/w%	
Yeast extract	2.5 w/w%	-	2.5 w/w%
Urea	-	0.3	0.3
KH_2PO_4	5	2	2
$(NH_4)_2SO_4$	-	1.4	1.4
NH_4NO_3	3	-	-
$(NH_4)_2HPO_4$	3	-	-
$MgSO_4$	0.5	0.3	0.3
NaCl	0.5	-	-
$CaCO_3$	0.5	-	-
$CaCl_2$	-	0.4	0.4
$FeSO_4$	-	0.005	0.005
$MnSO_4$	-	0.0016	0.0016
$ZnSO_4$	-	0.0014	0.0014
$CoCl_2$	-	0.002	0.002

2.5 Fungal cellulase extraction and assay

At the end of SSF, fungal cellulase was extracted and analysed. For each sample, 2 g of fermented substrate was mixed with 60 mL of sodium citrate buffer (50 mM, pH 4.8) in a blender (Ling Yang Frozen Machine Co., Hong Kong) for 10 seconds. The mixture was then centrifuged at 4°C, 10,000 *g* for 3 minutes to collect the clear supernatant as the crude enzyme sample. Total cellulase activity was determined by the filter paper activity (FPase) using the standardised NREL Laboratory Analytical Procedure (Adney and Baker, 1996).

In terms of the substrate used in this study, the FPase calculation was modified on the basis of dry weight of textile (Eq. 1).

$$\text{FPase activity (FPU/g)} = \frac{\text{FPase activity (FPU/mL)} \times \text{Total volume of the fungal extract (mL)}}{\text{Dry weight of the textile waste used in SSF (g)}}$$

Eq. (1)

2.6 Optimisation of physical factors by Response Surface Methodology (RSM)

For optimisation of SSF conditions, four physical parameters including pH, yeast extract concentration, inoculum size and moisture content were optimised via RSM with a four-factor-five-level central composite design (CCD) by Design-Expert® Software Version 8.0. Table 2 lists the value design of each factor. Cellulase activity (FPase) was set as the single response.

Table 2. Central composition design of four factors on SSF for cellulase production.

Numeric factor	Unit	Low value	High value	-alpha	+alpha
(A) pH	-	5	7	4	8
(B) Yeast extract	w/w %	1	4	0	5.5
(C) Inoculum size	spores g^{-1}	1.6E+007	4.6E+007	1E+006	6.1E+007
(D) Moisture	%	60	80	50	90

Based on the factors design above, a total of 30 runs with six centre points (set at the middle-value of each factor) were suggested by the software. Two replicates of each run were employed to verify any change in the estimation and experimental procedure. Accordingly, 60 samples were prepared with the designed conditions and then incubated for 9 days. To construct the model, all factors were coded using Eq. (2).

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \textbf{Eq. (2)}$$

Where X_i and X_0 are actual values of an independent variable in non-centre points and in centre points, respectively. The difference between X_i and X_0 was divided by step change value ΔX_i to gain the dimensionless value x_i.

With the obtained experimental result, a second order polynomial equation was suggested to describe the effect of each factor on cellulase production by linear, quadratic and cross product terms, as presented in Eq. (3)

$$Y = a_0 + \sum_{i=1}^{k} a_{ij} X_i + \sum_{i=1}^{k} a_{ij} X_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} X_i X_j + b$$

Eq. (3)

Where *Y* is cellulase activity as the single response, with *i* and *j* as linear coefficient and quadratic coefficient, respectively. The letter "*a*" is a regression coefficient and "*b*" is a random error. The number of factors is represented by "*k*".

2.7 Statistical analysis

The influence of each fermentation condition on cellulase production was evaluated by Analysis of Variance (ANOVA) based on F-test. Data processing and statistical analysis were performed by the software Design-Expert® 8.0.

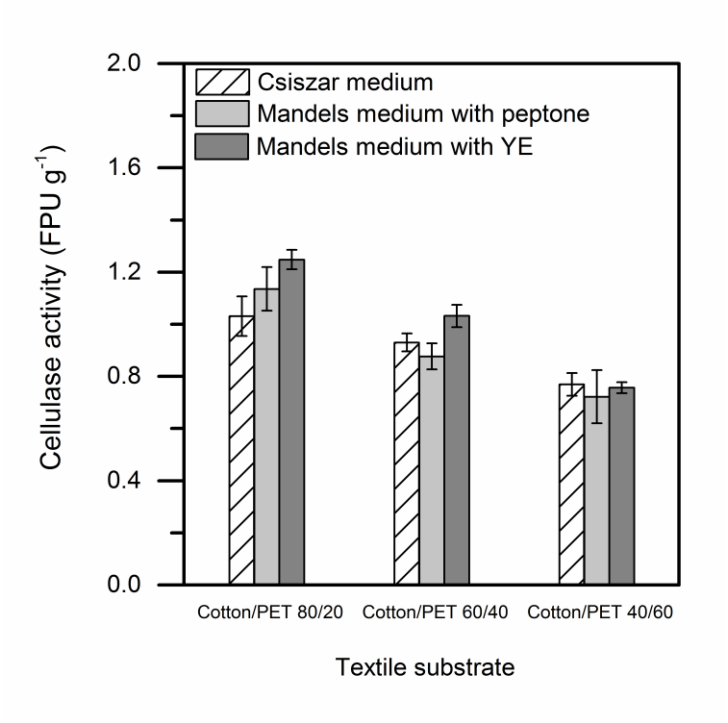
3. Results and discussion

3.1 Comparison of SSF on different cultivation media

The fermentation medium used in SSF usually consists of carbon and nitrogen sources, phosphorus and mineral elements. Csiszar medium and Mandels medium are commonly used for cellulase production using lignocellulosic substrate (Xin and Geng, 2010). Cellulose in substrate serves as the essential carbon source to induce cellulase generation, whereas nitrogen source can also stimulate cellulase and fungal biomass production (Kachlishvili *et al*., 2006). The effect of different cultivation media on cellulase production varied outcomes with different substrates and fungal strains (Kachlishvili *et al*., 2006). Therefore, it is important to have a proper combination of medium, substrate and fungal strain to maximise cellulase production

in SSF. In order to select a suitable medium for SSF on textile substrate using *A. niger* CKB, Csiszar medium and Mandels medium with either peptone or yeast extract as nitrogen source were investigated under the same incubation conditions.

As shown in Table 1, the main differences between Csiszar medium and Mandels medium are their nitrogen sources, minerals and the presence of Tween 80. Peptone, YE and ammonium salts are the commonly used nitrogen sources in fermentation media. Kachlishvili *et al.* (2006) reported that peptone and $(NH_4)_2SO_4$ were the most suitable nitrogen sources for CMCase production by *P. dryinus* on beech leaves and wheat straw, respectively. In this study, peptone and yeast extract were separately used as nitrogen sources in Mandels medium for SSF on textile. The result presented in Figure 1 shows that the use of yeast extract as nitrogen source led to higher cellulase activity on all three types of cotton/PET textile blends. In comparison to Csiszar medium, Mandels medium (with YE) generated higher cellulase activity with cotton/PET 80/20 and 60/40 blends as substrates. This could be attributed to the acceleration by supplementary trace elements (Fe, Mn, Zn, Co), which are cofactors of cellulase and supporting nutrients to fungal growth (Deswal *et al.*, 2011). Besides, Tween 80 in Mandels medium acts as a surfactant to improve the permeability of fungal cell membrane, which could enhance cellulase secretion (Ahamed and Vermette, 2008). Accordingly, Mandels medium with YE as nitrogen source was selected in SSF using textile waste, and the highest cellulase activity was 1.24 FPU g^{-1} obtained from cotton/PET 80/20 blend.



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241 **Figure 1.** Solid state fermentation on six types of textiles using various cultivation
242 media.

243

244 3.2 SSF under different temperature conditions

245 Incubation temperature is a fungal-dependent parameter that affects cellulase
246 production in SSF. For instance, *Aspergillus sp.* cultivation on rice grass was
247 suggested to be conducted at 32°C while *Fomitopsis sp.* was cultivated on wheat bran
248 at 30°C (Deswal *et al.*, 2011; Liang *et al.*, 2012). In general, the optimal temperature
249 for cellulase production by various fungi normally falls within a range of 25-34°C
250 (Gautam *et al.*, 2011). According to the study of Javed and Khan (2006), 30°C was
251 the optimised temperature for the fungal growth of *A. niger* species. In this study,
252 three types of cotton/PET blended textile waste were used as substrates to explore the
253 suitable temperature condition for cellulase production. *A. niger* CKB was cultivated
254 on each type of textile at 25°C, 28°C, 30°C, 32°C and 35°C separately, with 75%

moisture and 3×10^7 spores g^{-1} inoculum size for 9 days. The result of cellulase activities was determined as shown in Figure 2, from which the effect of different temperatures on the three types of textile shows similar pattern. SSF at 25°C limited cellulase activity to a low level. As temperature raised to 28-30°C, the result was significantly improved. Enzyme activities obtained at 28°C and 30°C were very similar. Higher temperatures at 32-35°C presented an obvious inhibition on cellulase production. This is mainly because that higher temperature led to rapid loss of moisture content from the fermentation substrate, hence the dry environment impaired fungal growth and metabolic activity. The results pinpointed the optimal temperature condition of 28°C for cellulase production on textile waste.

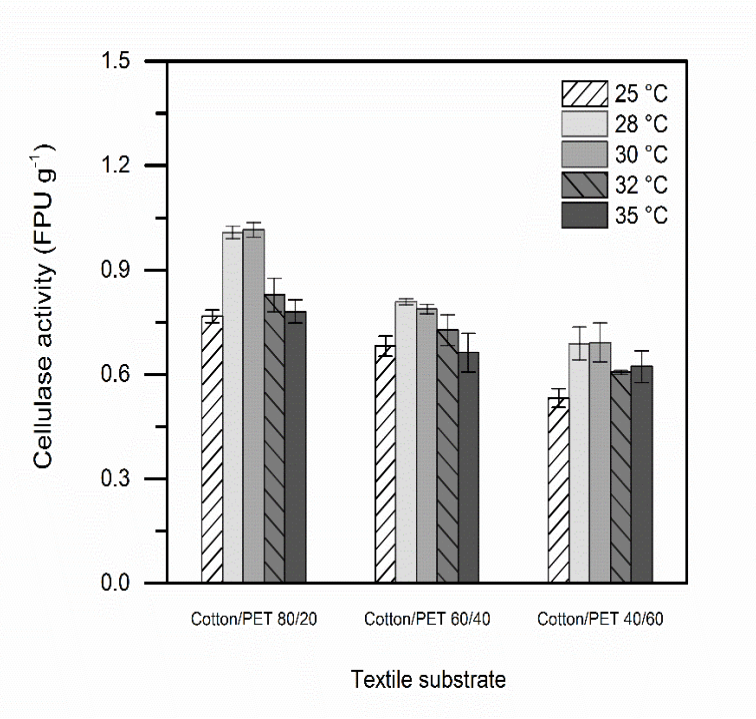


Figure 2. Cellulase production on textile waste at different temperatures.

3.3 Optimisation via Response Surface Methodology

Results in Section 3.1-3.2 indicated that the highest cellulase activity was obtained from 80/20 cotton/PET blend. Therefore, this textile was used as substrate in the subsequent investigation. SSF conditions including pH, yeast extract concentration, inoculum size and moisture content were optimised by RSM. Experiments were conducted in 30 runs according to the four-factor-five-level Central Composite Design (CCD). The specific condition of each run and the corresponding responses are listed in Table 3. Six runs at the centre point were bolded.

279 **Table 3.** Results of CCD in RSM and the corresponding responses for optimisation of

280 SSF conditions.

Run	pH	YE * (w/w %)	Inoculum size (spores g^{-1} textile)	Moisture (%)	Cellulase activity (FPU g^{-1})
1	7.00	4.00	4.60E+07	80.00	1.25
2	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.24**
3	4.00	2.50	3.10E+07	70.00	1.17
4	5.00	4.00	1.60E+07	60.00	0.73
5	6.00	2.50	1.00E+06	70.00	1.12
6	7.00	4.00	4.60E+07	60.00	0.85
7	6.00	5.50	3.10E+07	70.00	0.70
8	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.25**
9	7.00	1.00	4.60E+07	80.00	1.48
10	7.00	1.00	1.60E+07	60.00	0.76
11	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.22**
12	7.00	4.00	1.60E+07	60.00	0.61
13	6.00	2.50	3.10E+07	50.00	0.57
14	5.00	4.00	4.60E+07	60.00	0.91
15	8.00	2.50	3.10E+07	70.00	1.02
16	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.32**
17	7.00	1.00	4.60E+07	60.00	0.95
18	5.00	1.00	4.60E+07	60.00	0.78
19	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.28**
20	**6.00**	**2.50**	**3.10E+07**	**70.00**	**1.33**
21	6.00	2.50	6.10E+07	70.00	1.37
22	6.00	2.50	3.10E+07	90.00	1.04
23	5.00	1.00	1.60E+07	80.00	1.06
24	5.00	1.00	4.60E+07	80.00	1.05
25	5.00	4.00	1.60E+07	80.00	1.18
26	7.00	1.00	1.60E+07	80.00	1.08
27	7.00	4.00	1.60E+07	80.00	0.93
28	5.00	1.00	1.60E+07	60.00	0.75
29	5.00	4.00	4.60E+07	80.00	1.15
30	6.00	0.00	3.10E+07	70.00	0.57

281 *YE: yeast extract

282

The results show that cellulase activity obtained from cotton/PET 80/20 reduced over a range of 0.57-1.47 FPU g^{-1}. The condition in centre points resulted in cellulase activity of 1.22-1.33 FPU g^{-1}. Upon the result of response, a second-order polynomial model was constructed using Eq. (3) and Eq. (4). The ANOVA of each coded factor and interactions are listed in Table 4. The model F-value of 12.25 implies that the polynomial model was significant. There was statistically a 0.01% chance that this large F-value of model could occur due to noise. The effects of variables (A) pH, (B) yeast extract, (C) inoculum size and (D) moisture, along with interactions among these variables on cellulase activity were also evaluated by ANOVA.

Table 4. ANOVA of quadratic polynomial model for cellulase production from textile waste.

Source	Sum of squares	df	F-value	*p*-value Prob > F	Significance
Model	1.72	14	12.25	<0.0001	Significant
A	4.778E-006	1	0.00048	0.9829	
B	4.431E-003	1	0.44	0.5167	
C	0.14	1	12.68	0.0021	Significant
D	0.60	1	59.30	< 0.0001	Significant
AB	0.057	1	5.70	0.0305	Significant
AC	0.060	1	5.97	0.0274	Significant
AD	5.638E-003	1	0.56	0.4654	
BC	8.220E-004	1	0.082	0.7788	
BD	1.172E-004	1	0.012	0.9154	
CD	7.187E-005	1	0.0072	0.9337	
A^2	0.032	1	3.14	0.0969	
B^2	0.60	1	59.84	< 0.0001	Significant
C^2	1.127E-004	1	0.011	0.9171	
D^2	0.32	1	32.15	< 0.0001	Significant

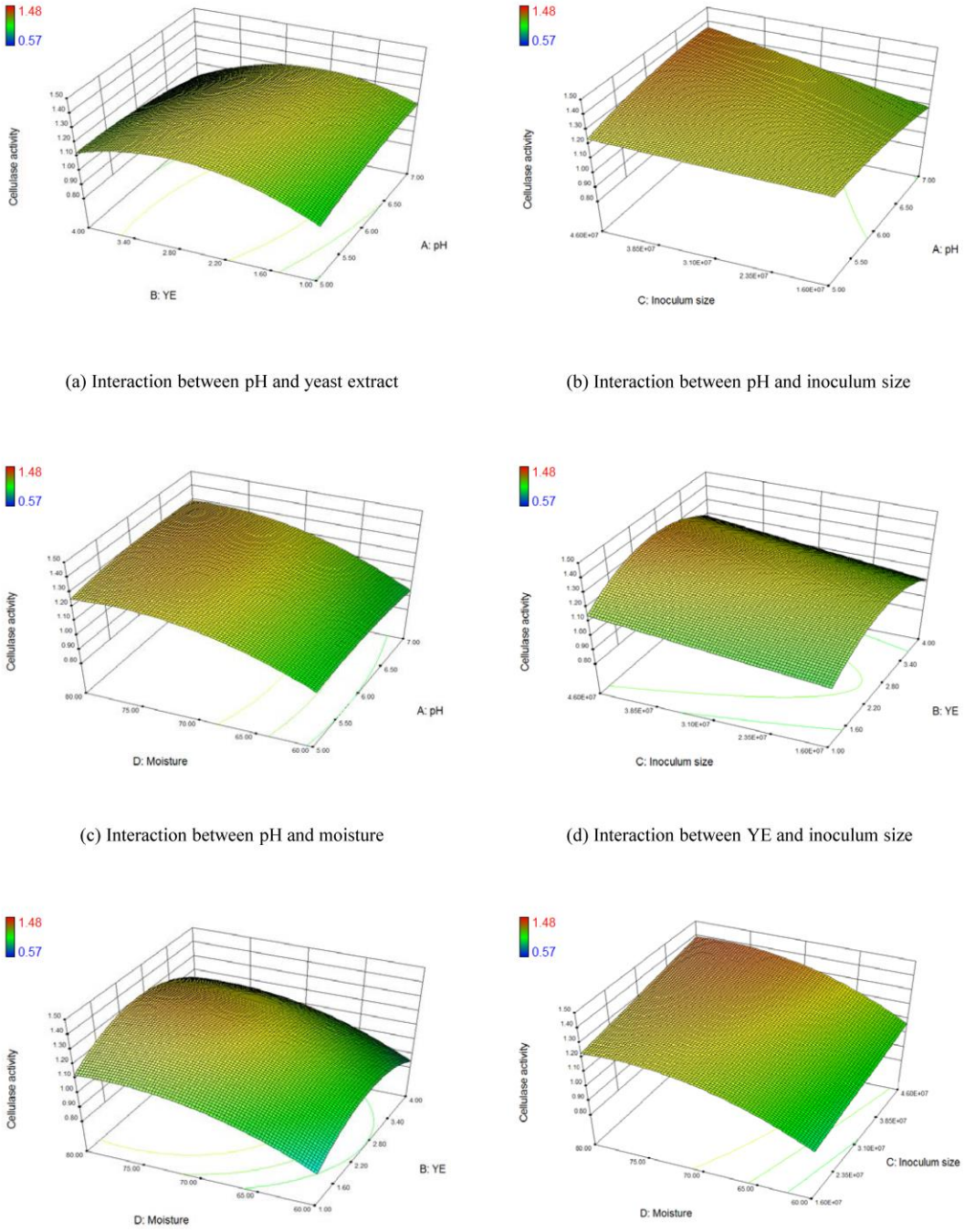
The ANOVA results indicate that linear (C, D), interaction (AB, AC) and quadratic (B^2, D^2) terms were statistically significant with *p*-values less than 0.0500. It means that these terms are the main affecting factors in regard to cellulase activity from SSF on textile waste. The model equation in terms of actual value of variables and response is described in Eq. (4).

$$Cellulase\ activity = -6.0143 + 0.24656 \times pH + 0.62448 \times YE - 2.17814\times10^{-8} \times Inoculum\ ratio + 0.15584 \times Moisture - 0.0399 \times pH \times YE + 4.08083\times10^{-9} \times pH \times Inoculum\ ratio + 1.87716\times10^{-3} \times pH \times Moisture + 3.1856\times10^{-10} \times YE \times Inoculum\ ratio - 1.80423\times10^{-4} \times YE \times Moisture + 1.41297\times10^{-11} \times Inoculum\ ratio \times Moisture - 0.033764 \times pH^2 - 0.074587 \times YE^2 + 8.97195\times10^{-18} \times Inoculum\ ratio^2 - 1.08093\times10^{-3} \times Moisture^2 \quad \textbf{Eq. (4)}$$

The coefficient of determination (denoted as R^2) of the equation was 0.9196 and the adjusted R^2 was 0.8445, indicating that the fitted linear, interaction and quadratic terms could elucidate 84.45% of the variation. The signal-to-noise ratio was measured by Adequate Precision, which is desired to be higher than 4. The constructed model ratio of 12.795 indicated an adequate signal so that the model can be used to navigate the design space.

The interactions between any two variables were depicted by response surface plots (3D) in Figures 3, with other factors fixed at the centre point values. Figure 3a shows the response surface curve of the resultant cellulase activity in response to changes in pH and yeast extract concentration, while the initial moisture content and inoculum size were maintained at 70% and 3.1×10^7 spore g^{-1}, respectively. As shown in Figure 3a, cellulase activity reached a maximum at pH 5.5-6.5 and yeast extract concentration of 2.20-3.40 w/w%. Figure 3b shows the significant interaction between

pH and inoculum size. As inoculum size and pH increased, cellulase activity grew from 1.10 to 1.30 FPU g^{-1}. In contrast, low cellulase activity was obtained at limited inoculum size (1.60×10^7 spore g^{-1}) and high pH value (> 7.0). At high levels of inoculum size, pH range of 5.5-7.0 slightly affected cellulase activity. While under neutral pH conditions (6.5-7.0), cellulase activity were sensitive to inoculum size. The response surface curve in Figure 3c indicates that the interactive effect of moisture and pH was obviously dominated by the former. When yeast extract concentration was 2.5 w/w% at centre point value, cellulase activity was gradually improved with increase of inoculum size (Figure 3d). Figure 3e shows an optimum response was reached under slightly higher moisture conditions (75-80%) in the interaction with yeast extract. Decrease of cellulase production occurred at both low and high levels of yeast extract, because of nutrient depletion and the inhibitory effect of nutrient surplus, respectively. Besides, although inoculum size and moisture both exhibited profound positive effects on cellulase production, the interaction was insignificant ($p > 0.05$). In spite of this, it could be noted from Figure 3f that the high levels of moisture (75-80%) and inoculum size (> 3.1×10^7 spore g^{-1}) enhanced cellulase activity to the peak value.



(a) Interaction between pH and yeast extract

(b) Interaction between pH and inoculum size

(c) Interaction between pH and moisture

(d) Interaction between YE and inoculum size

(e) Interaction between YE and moisture

(f) Interaction between inoculum size and moisture

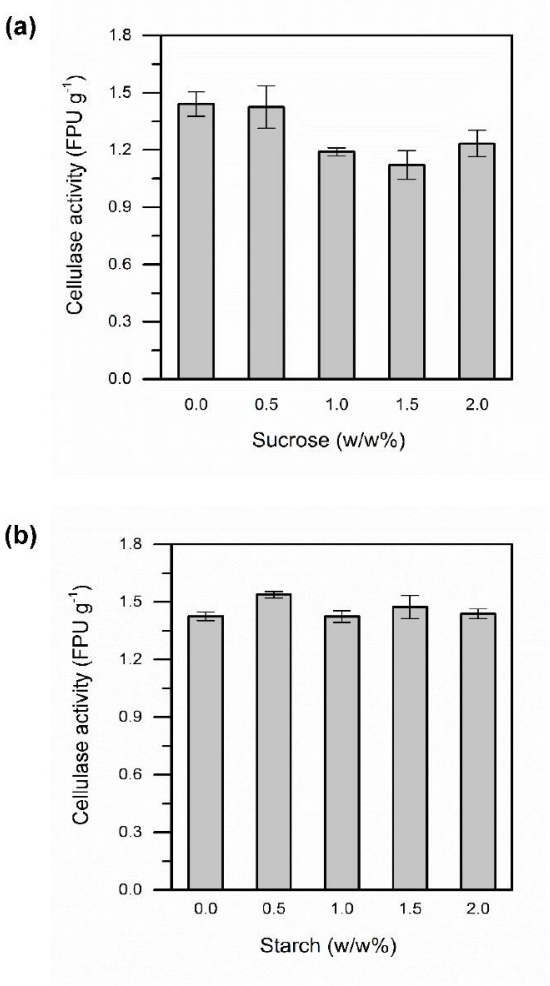
Figure 3. Response surface 3D plots of the interactions between various examined fermentation conditions.

In summary, the model suggested the optimum SSF condition at pH 7.29, yeast extract 2.24 w/w% and moisture of 78.53% with inoculum size of 4.60×10^7 spore g^{-1} for the maximised cellulase activity within the fixed range of each variable. Accordingly, cellulase activity of 1.48 FPU g^{-1} was predicted. Hence, the suggested condition was tested with different inoculum sizes of 1.60×10^7, 3.10×10^7 and 4.60×10^7 spore g^{-1}, resulting in cellulase activities of 1.13, 1.44 and 1.46 FPU g^{-1}, respectively. This revealed that under suitable conditions, adequate inoculum size is essential to high cellulase activity. However, this could not be linearly improved with further increase of inoculum size.

Therefore, based on the results above and by considering the experimental feasibility, the optimum SSF condition for cellulase production from textile waste was suggested to be pH 7.29, 2.24 w/w% yeast extract with a moisture content of 78% and an inoculum size of 3.10×10^7 spore g^{-1}. Fungal cellulase activity was improved by 16-20% from 1.20-1.24 FPU g^{-1} to 1.44 FPU g^{-1}.

3.4 Cellulase activity improvement by supplementary carbon sources

In order to improve fungal cellulase activity, sucrose and starch were added as supplementary carbon sources to enhance the fungal growth and enzyme production. Different loading ratios (0, 0.5, 1.0, 1.5, 2.0 w/w%) of sucrose/starch were added in SSF medium on the textile of cotton/PET 80/20. The optimum incubation conditions (from Section 3.2-3.3) of 9 days were applied in duplicate. The resultant cellulase activity is depicted in Figure 4.



365

366 **Figure 4.** Effect of supplementary (a) sucrose and (b) starch on cellulase production
367 from textile waste.

368

369 The results reveal that the addition of sucrose did not improve cellulase activity.
370 Addition of sucrose as an inducer at relatively higher loading ratios (1.0-2.0 w/w%)
371 even exhibited an inhibitory effect on cellulase production. In comparison,
372 supplementary starch enhanced cellulase activity to a certain extent. The significance
373 of variances caused by different starch dosages was judged by ANOVA (Table 5).
374 The addition of starch by 0.5, 1.5 and 2.0 w/w% led to a positive effect on cellulase

activity. ANOVA result pointed out that when 0.5 w/w% starch was added, the corresponding cellulase activity improvement (from 1.43 to 1.53 FPU g^{-1}) was significant as *p*-value was lower than 0.05. In contrast, variances caused by other loading ratios could have occurred due to noise with high probability (40.24% - 98.69%). Therefore, the supplement of 0.5 w/w% starch was suggested in SSF for cellulase production from textile waste.

Table 5. Effect of supplementary starch on cellulase production from textile waste by ANOVA.

Starch addition (w/w%)	Cellulase activity (FPU g^{-1})	Standard deviation	F value	*p* value Prob > F
0.0	1.43	0.0212	N/A	N/A
0.5	1.53	0.0170	35.22	0.0272
1.0	1.42	0.0318	3.42E-04	0.9869
1.5	1.47	0.0608	1.11	0.4024
2.0	1.44	0.0490	0.31	0.6348

3.5 Effect of inducer on cellulase production from textile waste

Natural inducers of fungal cellulase generation have been investigated since 1957 in Mandel and Reese's study (Mandels and Reese, 1957). They suggested that cellulase could only be produced on glucose, lactose, cellubiose and cellulose. Lactose and basic celluloses consisting of anhydroglucose units with β-1-4-glycosidic linkage were proposed as excellent inducers to stimulate cellulase secretion towards breaking β-1-4-glycosidic bonds to obtain the monomeric glucose. Therefore, lactose has been applied as an inducer in the commercial production of cellulase (Sukumaran *et al.*, 2005).

In this study, lactose and basic celluloses including Avicel, sodium carboxymethyl cellulose (sodium CMC) and filter paper scrap (Whatman No.1, 100% cellulose) were employed as inducers in SSF on textile waste. Inducers were added separately in gradients from 0.5 to 5.0 w/w%. SSF was performed on cotton/PET 80/20 blend using the optimum condition with 0.5 w/w% starch for 9 days. A control group was set without any inducer. All conditions were conducted in duplicate and the harvested cellulase activities are depicted in Figure 5.

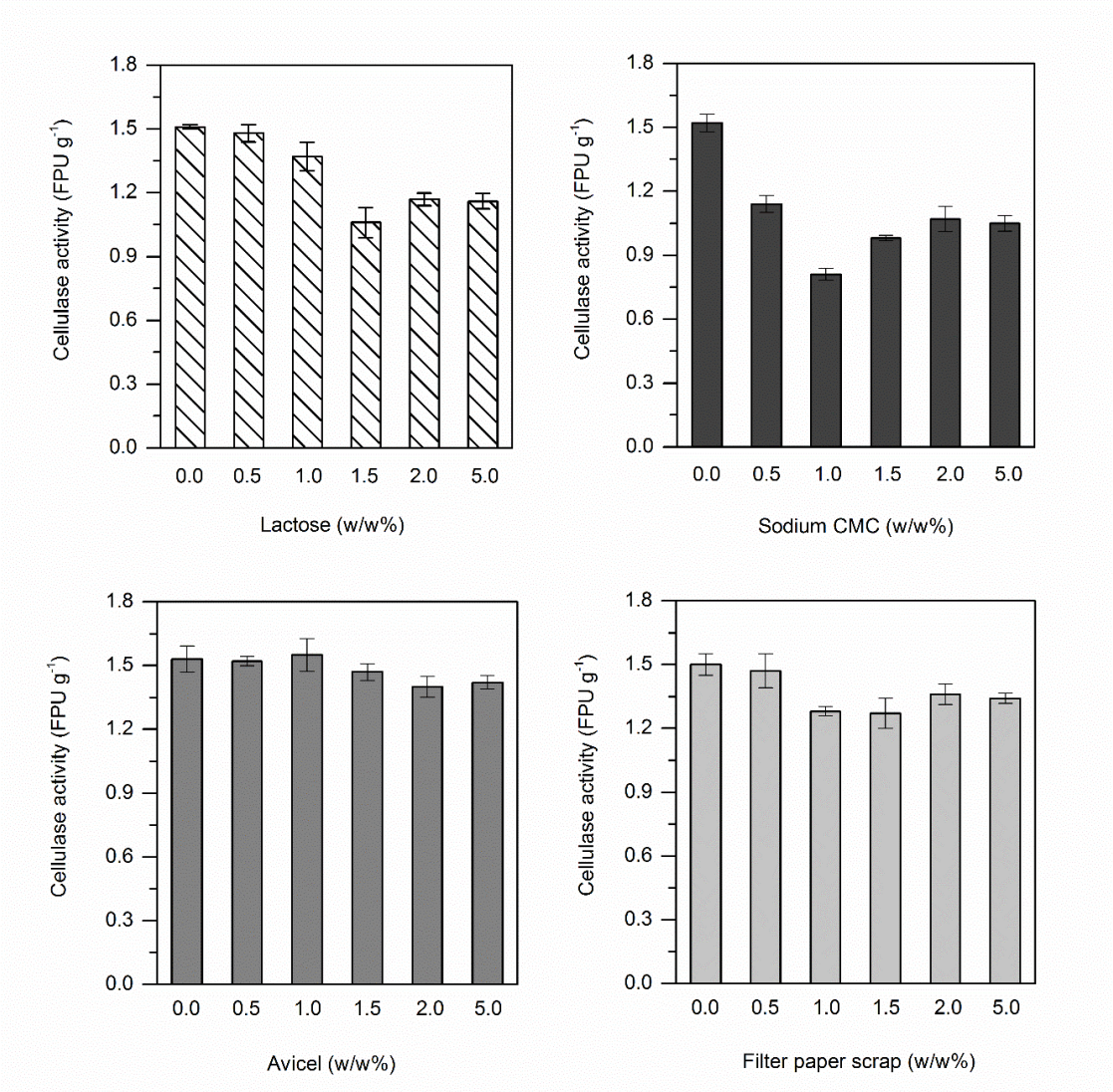


Figure 5. Effect of inducers on cellulase production from textile waste (80/20 cotton/PET blend).

The results show that the addition of lactose, sodium CMC or filter paper scraps failed to enhance cellulase activity to a higher level. In these three sets, the highest cellulase activities were obtained from the control group (without inducer). Sodium CMC loaded at weight ratios higher than 1% exhibited significant inhibitory effect on cellulase production, resulting in a reduction of enzyme activity from 1.52 FPU g^{-1} to 0.81-1.07 FPU g^{-1}. In comparison, with 1.0 w/w% of Avicel as an inducer, cellulase activity increased slightly from 1.53 to 1.55 FPU g^{-1}.

The insignificant inducing effect could be attributed to several possible reasons. Firstly, the metabolic activity of *A. niger* CKB cannot be simply induced through direct addition of Avicel/basic cellulose into fermentation medium. Secondly, the heterogeneous substrate by mixing insoluble inducers (*e.g.* sodium CMC, filter paper scraps) with textile fabric is not suitable for fungal growth. For instance, the addition of sodium CMC in textile substrate led to high viscosity of mixture and thereby inhibited aerobic condition along with fungal colonisation. Besides, the time course of cellulase production might have been affected by supplementary starch and the inducer. Therefore, the enzyme activity collected on day 9 could not clearly distinguish the variance.

3.6 Time course of cellulase activity under optimised SSF conditions

The time course of fungal cellulase production from textile waste was investigated in our previous study (Hu *et al*., 2017). The total cellulase activity increased from day 3 and reached peak value on day 9 in SSF. In this study, the cultivation conditions were optimised, in which it essentially affected the cellulase activity of fungal enzyme product. Thus, the corresponding time course deserves further exploration in order to

429 determine the production profile of cellulase activity. *A. niger* CKB was incubated on
430 the textile of cotton/PET 80/20 under different conditions in Set A, B and C as listed
431 in Table 6.

432 **Table 6.** Fungal cellulase production under different SSF conditions.

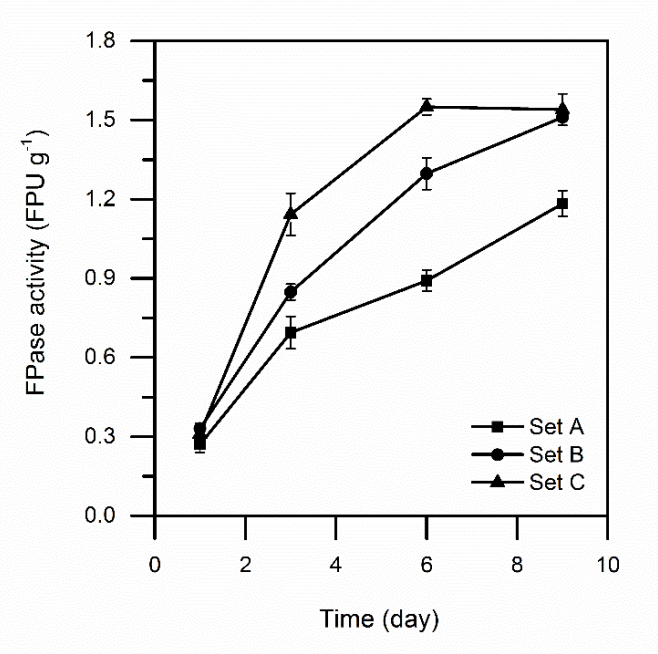
	Set A	Set B	Set C
Moisture condition (%)	75.0	78.0	78.0
Inoculum size (10^7 spores g^{-1})	3.1	3.1	3.1
pH	6.0	7.2	7.2
Yeast extract (w/w%)	2.50	2.24	2.24
Starch (w/w%)	0.0	0.5	0.5
Avicel (w/w%)	0.0	0.0	1.0

433

434 The conditions applied in Set A are the middle-value of each factor, without
435 additional carbon source or inducer. Conditions in Set B were the optimal solution
436 from RSM according to the results in Section 3.3 and with starch (0.5 w/w%) as a
437 supplementary carbon source. Furthermore, Avicel (1.0 w/w%) was supplied as an
438 inducer in Set C. All sets were tested in duplicate at 28°C. Figure 6 shows the
439 cellulase activity (FPase) profile of Set A, B and C.

440 It was found that under optimal SSF conditions, cellulase activity in Set B and C
441 increased from day 1 at higher efficiency as compared to the status in Set A. The
442 addition of Avicel (in Set C) further improved cellulase activity and led to the
443 maximum of 1.56 FPU g^{-1} on day 6. Therefore, the combination of optimum
444 fermentation conditions, supplementary starch and Avicel indeed enhanced fungal
445 cellulase production, which was consequently accomplished in reduced incubation
446 period (from 9 days to 6 days) with 25.8% increase of total cellulase activity. The

447 production efficiency of fungal cellulase from textile waste was significantly
448 improved by 88.7%.



449

450 **Figure 6.** Time courses of cellulase production under different SSF conditions.

451

452 Upon the optimum SSF, the overall process of the novel circular textile waste
453 approach in the project entitled "Textile Waste Recycling by Biological Method" is
454 described in Figure 7. Textile waste from manufacturers and from fermented substrate
455 in SSF was inputted as raw materials. The fungal cellulase (1.56 FPU g^{-1}) was
456 employed as an enzyme source in textile waste hydrolysis, which led to a glucose
457 recovery yield of 70.2% within 96 h. The resultant hydrolysate was a glucose-rich
458 stream separated from the remaining solid (*i.e.* polyester) by filtration. This glucose-
459 rich stream could be utilised as a generic feedstock in microbial fermentation for the
460 production of value-added products via product refining. According to the
461 experimental results, around 624 kg of glucose and 200 kg of PET could be recovered
462 from 1,000 kg of textile waste (cotton/PET 80/20). The recovered PET was processed
463 into fibres by melting spinning towards textile applications for the first time by Li *et*

al. As compared to recovering PET merely by paper filtration (Shen *et al.*, 2013; Jeihanipour *et al.*, 2010), melting spinning waste PET into textile fibre would definitely increase its commercial value. Therefore, through the proposed biorefining approach, textile waste can be efficiently recycled to value-added products, which can benefit the circular economy. The economic performance and technical feasibility of the overall process at pilot scale is currently under evaluation in our group.

4. Conclusions

This study illustrated the optimisation of fungal cellulase production from textile waste using experimental design. Different cultivation media were compared on three types of cotton/PET blended textile waste. Typical fermentation affecting factors were optimised through one-variable method and Response Surface Methodology, which suggested the optimum SSF conditions by using Mandels medium with yeast extract (2.24 w/w%), the moisture content of 78%, the inoculum size of 3.10×10^{7} spore g^{-1} and pH 7.29 at 28°C. The addition of starch (0.5 w/w%) and Avicel (1 w/w%) further increased cellulase activity to 1.56 FPU g^{-1} with significantly improved production efficiency. The outcomes reported in this study could contribute to an innovative circular textiles approach, which enables the transition from the current linear to stronger circular economy model.

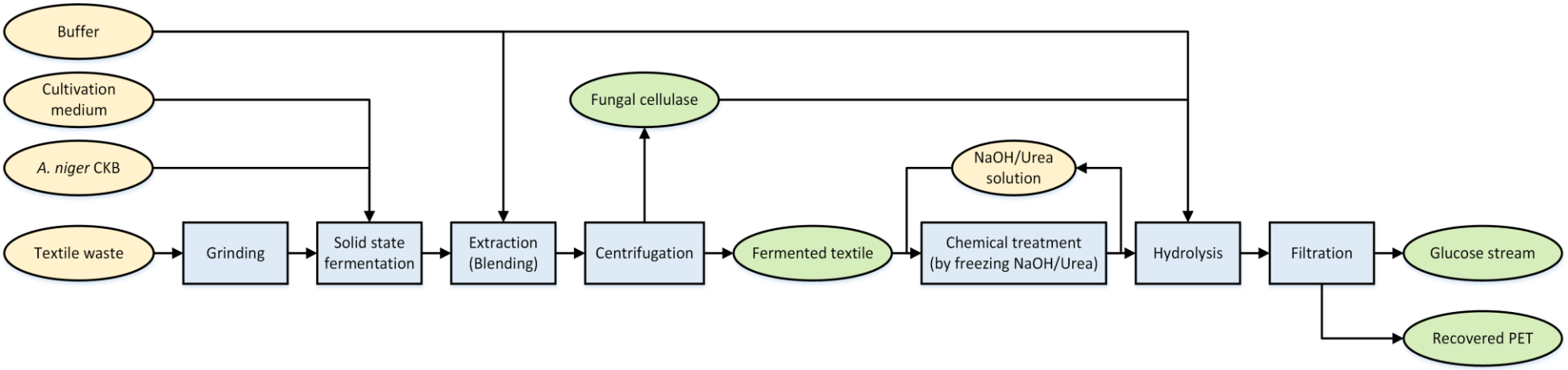


Figure 7. Process scheme of the textile waste recycling approach through biological method.

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